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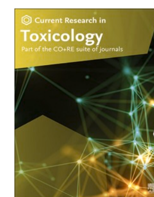

Sesquiterpene-evoked phytochemical toxicity in PC12 neuronal cells reveals a variable degree of oxidative stress and alpha-tocopherol and glutathione-dependent protection

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ABSTRACT

Phytochemicals are often promoted generally as antioxidants and demonstrate variable levels of reactive oxygen species (ROS) sequestration in vitro, which attributes to their neuroprotective bioactivity. Sesquiterpenes from cannabis and essential oils may demonstrate bifunctional properties towards cellular oxidative stress, possessing pro-oxidant activities by generating ROS or scavenging ROS directly. Sesquiterpenes can also oxidize forming sesquiterpene oxides, however the relative contribution they make to the bioactivity or cytotoxicity of complex botanical extracts more generally is unclear, while selected cannabis-prevalent terpenes such as β-caryophyllene may also activate cannabinoid receptors as part of their biological activity. In the present study, we investigated selected sesquiterpenes β-caryophyllene and humulene and their oxidized forms (β-caryophyllene oxide and zerumbone, respectively) against established antioxidants (ascorbic acid, α-tocopherol, and glutathione) and in the presence of cannabinoid receptor 1 and cannabinoid receptor 2 antagonists, to gain a better understanding of the molecular and cellular mechanisms of neuroprotection versus neurotoxicity in semi-differentiated rat neuronal phaeochromocytoma (PC12) cells. Our results demonstrate that the sesquiterpenes β-caryophyllene, humulene and zerumbone possess concentration-dependent neurotoxic effects in PC12 cells. Both β-caryophyllene- and humulene-evoked toxicity was unaffected by CB1 or CB2 receptor antagonism, demonstrating this occurred independently of cannabinoid receptors. Both glutathione and α-tocopherol were variably able to alleviate the concentration-dependent loss of PC12 cell viability from exposure to β-caryophyllene, humulene and zerumbone. During 4-hour exposure to sesquiterpenes only modest increases in ROS levels were noted in PC12 cells, with glutathione co-incubation significantly inhibiting intracellular ROS production. However, significant increases in ROS levels in PC12 cells were demonstrated during 24-hour incubation with either antioxidants or sesquiterpenes individually, and with additive toxicity exhibited in combination. Overall, the results highlight a concentration-dependent profile of sesquiterpene neurotoxicity independent of cannabinoid receptors and dissociated from the formation of reactive oxygen species as a marker or correlate to the loss of cell viability.

Introduction

Oxidative stress is most notably caused by the production and accumulation of reactive oxygen species (ROS) and has been recognized as a key contributor to many neurodegenerative diseases, including Alzheimer's disease (Martins et al., 2018). ROS are highly reactive, chemically unstable, and cause oxidative damage to cells resulting in lipid peroxidation, nucleic acid oxidation and mitochondrial dysfunction, but levels are kept relatively low by cellular antioxidants (Markowitz et al., 2007). Direct antioxidants can scavenge ROS and are redox active, while indirect antioxidants can induce cytoprotective enzymes and intracellular antioxidants (Markowitz et al., 2007). Conversely, when antioxidant defenses exceed oxidant production, a type of 'reductive stress' can occur; this may occur in the early stages of

Abbreviations: AD, Alzheimer's disease; ROS, Reactive oxygen species; BCP, β-caryophyllene; CB1R, Cannabinoid receptor 1; CB2R, Cannabinoid receptor 2; *C. sativa*, *Cannabis Sativa* L.; Humulene, HUM; BCPO, β-caryophyllene oxide; ZER, Zerumbone; AA, Ascorbic acid; GSH, Glutathione; PC12, Semi-differentiated rat neuronal phaeochromocytoma cells; *t-BHP*, *tert*-butyl hydroperoxide; Keap1, Kelch-like ECH-associated protein 1; Nrf2, Erythroid 2–related factor 2; ECN, 7β-(3-ethyl-*cis*-crotonoyloxy)-1α-(2-methylbutyryloxy)-3,14-dehydro-Z-notonipetranone; HSP70, Heat shock protein 70.

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neurodegenerative disease before symptomology and may be a preceding stage towards subsequent oxidative stress which compounds further neurotoxicity (Lloret et al., 2016).

Highly aromatic plants have been used for their component essential oils for millennia and are now emerging as a source of natural phytochemicals for redox homeostasis (Amorati et al., 2013). Essential oils as natural bioactive alternatives can serve as bifunctional antioxidants; however their constituents also possess pro-oxidant activities by promoting and generating ROS or by inhibiting antioxidant defense systems (Bartikova et al., 2014). Terpenes are the main constituents of essential oils that are synthesized as aliphatic compounds in various proportions from numerous aromatic plants for their antimicrobial activities, herbivore deterrence and reproductive success (Buchbauer and Ilic, 2013). Terpenes are classed into five categories (monoterpenes, sesquiterpenes, diterpenes, triterpenes and tetraterpenes) according to the number of isoprenoid units. Sesquiterpenes are a class of terpenes containing 3 isoprene units (5-carbon building blocks), and are mostly small lipophilic molecules which can readily oxidize forming terpenoids or isoprenoids (Andre et al., 2016). Sesquiterpenes are rapidly bioavailable though pulmonary, oral, and dermal administration (Jäger and Höferl, 2020), can effectively penetrate the blood brain barrier (Sánchez-Martínez et al., 2022) and are generally recognized as safe to human health (Schmitt et al., 2016).

Aside from phytocannabinoid production, *Cannabis Sativa* L. (*C. sativa)* is a botanical source of terpene-rich essential oils (Vuerich et al., 2019; Zuk-Golaszewska and Golaszewski, 2018), with some strains synthesizing up to 35 % β-caryophyllene (BCP) and up to 8.3 % humulene (HUM) (Kwaśnica et al., 2020; Richter et al., 2021). Burgeoning access to medicinal and recreational cannabis has driven advancement of cannabis extract production from predominantly dried *C. sativa's* inflorescences (Meehan-Atrash et al., 2019). While most *C. sativa* extracts are typically heated to decarboxylate the acidic cannabinoids to the more bioactive form, the effect of manufacturing, storage and decarboxylation can decrease sesquiterpene content and accelerate the development of their oxidized forms (Milay et al., 2020). Furthermore, sesquiterpene oxide's relative contribution to the bioactivity or cytotoxicity of complex botanical extracts more generally is unclear; considering the escalating use of cannabis products of varying formulations containing such sesquiterpenes and potentially their oxidized forms, it's desirable that further neurological bioactivity and safety is explored for human health (Baldovinos et al., 2023; Guo et al., 2021).

Therefore, in the present study, we investigated the neuronal bioactivity of BCP and HUM and their oxidized forms ((β-caryophyllene oxide (BCPO) and zerumbone (ZER), respectively)) (Fig. 1), alone and against established antioxidants ((ascorbic acid (AA), α-tocopherol, and glutathione (GSH)). Additionally, we profiled activity of BCP and HUM in the presence of cannabinoid receptor CB1R and CB2R antagonists, to gain a better understanding of the pharmacological, molecular and cellular mechanisms of neuroprotection versus neurotoxicity in semi-differentiated rat neuronal phaeochromocytoma cells (PC12), where BCP (but not BCPO or HUM) has been previously ascribed CB2 receptor agonist activity (Gertsch et al., 2008). PC12 cells are a robust and validated neuronal cell model for researching neurodegenerative disorders and are also intensively used to investigate ROS biochemical pathways involved in cell death and neuroprotection (Liu et al., 2020; Wiatrak et al., 2020). In addition, we have previously demonstrated that Ordway PC12 cell sub-clone expresses both cannabinoid receptor subtypes (Harvey et al., 2012), while another study showed BCP-evoked differentiation in PC12 cells independently of CB receptors, suggesting potentially wide-ranging bioactivity of such terpenes in this neuronal cell line (Santos et al., 2017).

Materials and methods

Reagents and chemicals

β-caryophyllene, humulene, β-caryophyllene oxide, zerumbone, thiazolyl blue tetrazolium bromide (MTT), thioflavin T, trypan blue, *tert*-butyl hydroperoxide (*t*-BHP), DMSO, Roswell Park Memorial Institute 1640 (RPMI) medium, non-essential amino acids (NEAA), penicillin/streptomycin, trypsin EDTA, foetal bovine serum (FBS), ascorbic acid, α-tocopherol, glutathione, AM251, SR144528 and phosphate buffered saline (PBS) were all supplied by Sigma-Aldrich (Sydney, Australia).

Preparation of terpenes, antioxidants, and cannabinoid CB1 and CB2 receptor antagonists

All terpenes, AM251, SR144528 and α-tocopherol were dissolved in ethyl alcohol (70 %) and vortexed until fully dissolved prior to dilution in sterile PBS to final working concentrations. Ascorbic acid and glutathione were dissolved in PBS and vortexed until fully dissolved prior to dilution in sterile PBS to final working concentrations.

Neuronal PC12 cell culture

The Ordway PC12 cell sub-clone was generously donated by Professor Jacqueline Phillips (Macquarie University, NSW, Australia) and exhibits a semi-differentiated neuronal phenotype with neuronal projections, making them phenotypically representative of neurons without requiring extensive differentiation (Dixon, Loxley, Barron, Cleary, & Phillips, 2005). PC12 cells were maintained in RPMI-1640 media with 10 % (v/v) foetal bovine serum (FBS), 1 % (v/v) penicillin/streptomycin and 1 % (v/v) non-essential amino acids at 37 °C with 5 % carbon dioxide (CO_2). Media was replenished every 2–3 days prior to seeding in 96 well plates at 3×10^4 cells per well in 100 μL media at 37 °C with 5 % CO_2 and equilibrated for 24 hrs prior to treatment.

Neuronal cell viability measurements

PC12 cell viability was measured using the thiazolyl blue tetrazolium bromide (MTT) assay, based on an established protocol (Marsh, Das, Ridell, & Smid, 2017). PC12 cells were pre-treated with either ascorbic acid (50 μM), α-tocopherol (100 μM), glutathione (1 mM), the CB1 receptor inverse agonist AM251 (100 nM), or CB2 receptor antagonist SR144528 (100 nM) for 15 min prior to incubation with

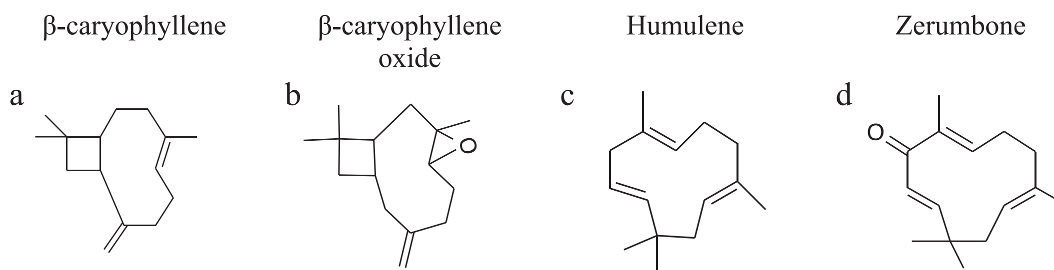

Fig. 1. Terpenes used in this study; (**a**) β-caryophyllene (BCP), (**b**) β-caryophyllene oxide (BCPO), (**c**) humulene (HUM) and (**d**) zerumbone (ZER).

β-caryophyllene, humulene, β-caryophyllene oxide, zerumbone (0–200 μM for 24 hrs) at 37 °C with 5 % CO_2. Vehicle-treated (control) cells had equivalent matched concentrations of solvent in wells (<1% ethanol v/v). After incubation, the media was aspirated and replaced with 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (100 μL). The cells were then incubated for a further 2 hrs at 37 °C with 5 % CO_2 before MTT solution was aspirated from all wells. DMSO was added to wells to lyse cells and absorbance was measured at 570 nm using a Synergy MX microplate reader (Bio-Tek, Bedfordshire, UK).

Measurement of intracellular ROS levels

Intracellular ROS levels were determined using DCFDA / H2DCFDA - Cellular ROS Assay Kit (Abcam; Australia) according to the manufacturer's instructions. Briefly, PC-12 cells were stained with DCFDA (20 μM) for 30 min at 37 °C in the dark. Cells were then washed with PBS and pretreated with either ascorbic acid (50 μM), α-tocopherol (100 μM), glutathione (1 mM), for 15 min prior to treatment with *tert*-butyl hydroperoxide (*t*-BHP) (50 μM and 200 μM), β-caryophyllene, humulene, β-caryophyllene oxide, and zerumbone (200 μM for 4 hrs and 24 hrs) in phenol red-free media for 4 hrs at 37 °C with 5 % CO_2 in the dark. Fluorescence intensity was subsequently measured with excitation/emission wavelengths at 485 nm / 535 nm in a BioTek Synergy Mx microplate reader (BioTek, Vermont, USA).

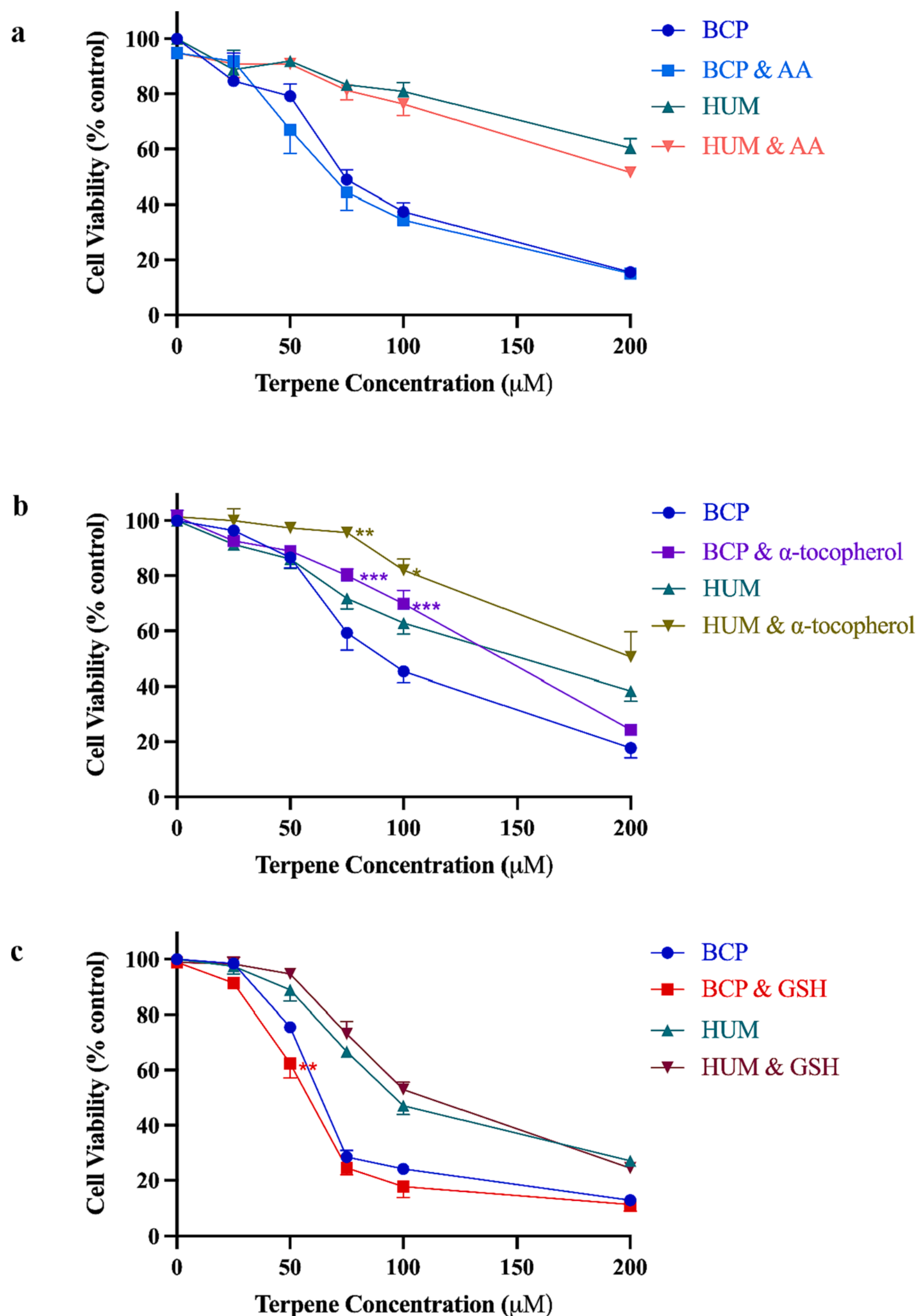

Fig. 2. MTT assay of PC12 neuronal cell viability following 24 hr incubation with β-caryophyllene (BCP) and humulene (HUM), alone and in the presence of (**a**) AA (50 μM), (**b**) α-tocopherol (100 μM) and (**c**) GSH (1 mM). α-tocopherol significantly inhibited loss of cell viability arising from both BCP and HUM incubation. ***P < 0.001 **P < 0.01, *P < 0.05 vs sesquiterpene. n = 4.

Data and statistical analysis

All data was analyzed for statistical significance using GraphPad Prism 8 for Mac (GraphPad Software, CA, USA). PC12 cell viability was analyzed following treatment with β-caryophyllene, humulene, β-caryophyllene oxide, zerumbone in the presence of the selected antioxidants using 2-way ANOVA, with Bonferroni's post-hoc test used to determine the significance of each terpene's effect at each neurotoxicant concentration. Each plate was an independent experiment (n) with an average of quadruplicate samples taken per plate. All data was represented as mean ± SEM and significance levels of $p < 0.05$ utilized throughout. ROS activity was analyzed via one-way ANOVA with a Dunnett's post-hoc test. Data analysis and graphs were performed in GraphPad Prism 8 (GraphPad Software, San Diego, USA).

Results

Effects of antioxidants on PC12 cell viability in the presence of β-caryophyllene and humulene

After 24 hr incubation a concentration-dependent loss of PC12 cell viability was demonstrated in the presence of both BCP and HUM (25 μM – 200 μM; Fig. 2a-c). There was significant enhancement of cell viability in the presence of α-tocopherol (100 μM) across concentrations of BCP and HUM at 75 μM and 100 μM (Fig. 2b), but neither ascorbic acid (AA; Fig. 2a) or glutathione (GSH; Fig. 2c) provided any mitigation of sesquiterpene-evoked neurotoxicity.

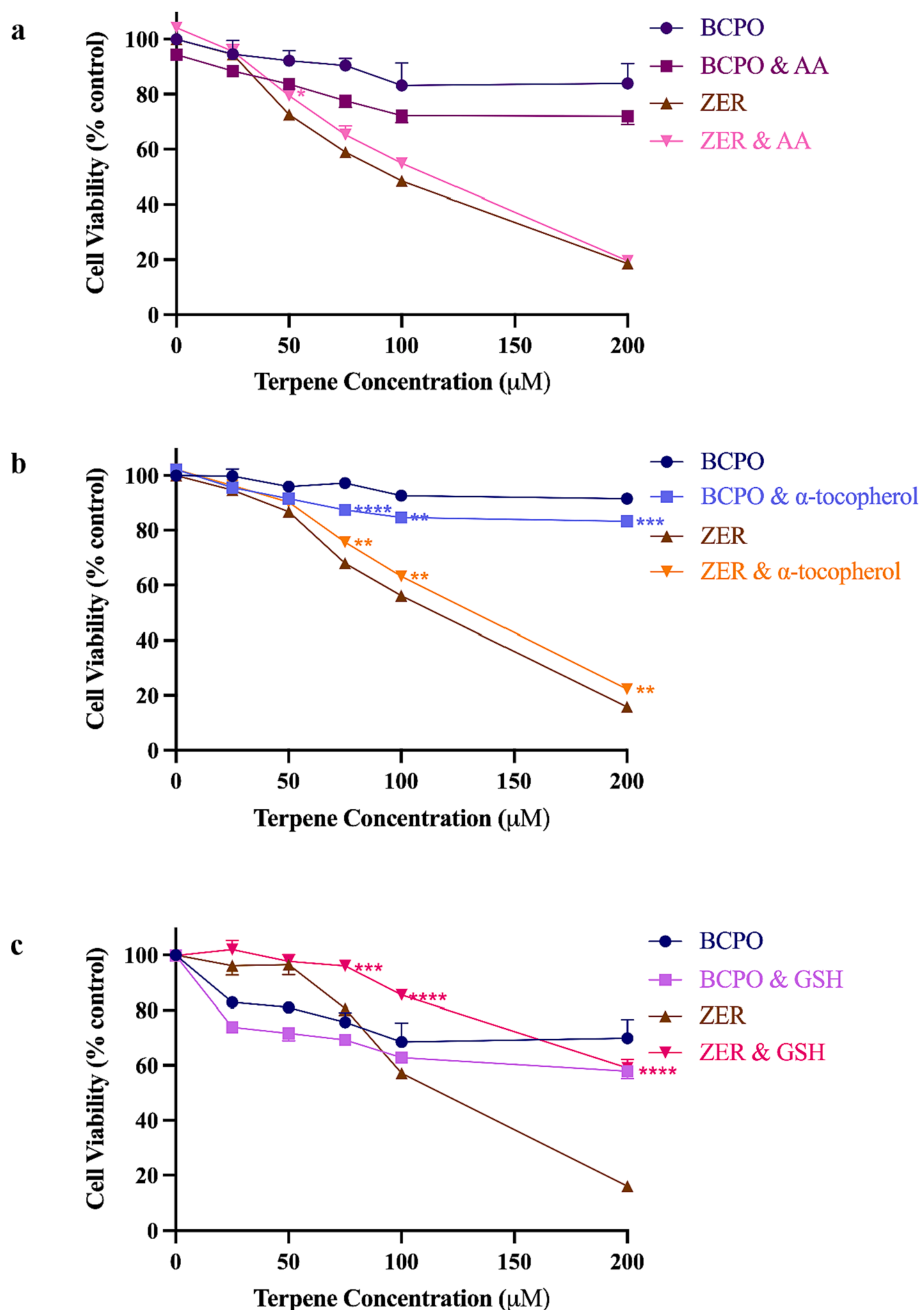

Fig. 3. MTT assay of PC12 neuronal cell viability following 24 hr incubation with β-caryophyllene oxide (BCPO) and zerumbone (ZER), alone and in the presence of (**a**) AA (50 μM), (**b**) α-tocopherol (100 μM) and (**c**) GSH (1 mM). GSH significantly inhibited loss of cell viability arising from ZER incubation, as did α-tocopherol to a lesser extent, while conversely α-tocopherol caused a small augmentation of BCPO toxicity. ****P < 0.0001 ***P < 0.001 **P < 0.01, *P < 0.05 vs vehicle. n = 4.

Effects of antioxidants on PC12 cell viability in the presence of β-caryophyllene oxide and zerumbone

A concentration-dependent loss of PC12 cell viability was demonstrated in the presence of zerumbone (ZER; 25–200 μM), while the loss of PC12 cell viability was minimal in response to β-caryophyllene oxide (BCPO; Fig. 3a-c). There was significant enhancement of cell viability in the presence of GSH (1 mM) across concentrations of ZER from 75 μM to 200 μM (Fig. 3c). Modest enhancement of cell viability was observed in the presence of α-tocopherol (100 μM) across concentrations of ZER from 75 μM to 200 μM (Fig. 3b) with a small but significant enhancement in toxicity in the BCPO treatment group with α-tocopherol (Fig. 3b).

Effects of CB1R and CB2R antagonist on PC12 cell viability in the presence of β-caryophyllene and humulene

A concentration-dependent loss of PC12 cell viability was again demonstrated in the presence of either BCP and HUM (50–200 μM; Fig. 4a-b); however no overall changes were observed in the presence of either the CB2 receptor antagonist SR155528 (100 nM; Fig. 4a) or CB1 receptor inverse agonist AM251 (100 nM; Fig. 4b), demonstrating that the loss of cell viability was independent of cannabinoid receptors.

Effects of antioxidants on reactive oxygen species in the presence of β-caryophyllene, humulene, β-caryophyllene oxide and zerumbone after short-term (4-hour) incubation

Of the sesquiterpenes tested, only humulene (HUM) significantly elevated DCFH-DA fluorescence as a measure in intraneuronal ROS in PC12 cells at 4 hrs (Fig. 5b). A modest but significant reduction in intracellular ROS was demonstrated from pretreatment with GSH before incubation with either BCP, HUM, BCPO and ZER (200 μM) for 4 hrs (Fig. 5a-d). AA and α-tocopherol had no further effects against all terpenes tested, indicating a modest, significant and more widespread antioxidant effect of GSH in the presence of any of the four sesquiterpenes at 4 hrs.

Effects of antioxidants on reactive oxygen species in the presence of β-caryophyllene, humulene, β-caryophyllene oxide and zerumbone after long-term (24-hour) incubation

After 24 hr incubation, significant increases in ROS were demonstrated from treatment with each sesquiterpene alone (Fig. 6a-d). Paradoxically, antioxidant treatment alone also significantly increased ROS generation after 24 hrs. In combination, sesquiterpenes and antioxidants further increased ROS generation, significantly for each terpene in the presence of α-tocopherol (Fig. 6a-d) and BCP and GSH (Fig. 6a).

Discussion

Neuronal cells are especially predisposed to oxidative stress because of their high oxygen consumption, low antioxidant defenses, abundance of unsaturated fatty acids and high iron content (Valko et al., 2007). Terpenes have been associated with cytotoxicity via targeting mitochondria to initiate various biochemical events ranging from inhibition of complexes I and II of the electron transport chain, decreasing adenosine triphosphate (ATP) and GSH levels (Usta et al., 2009) and can play a role in lipid peroxidation, oxidative damage and increased cytotoxic

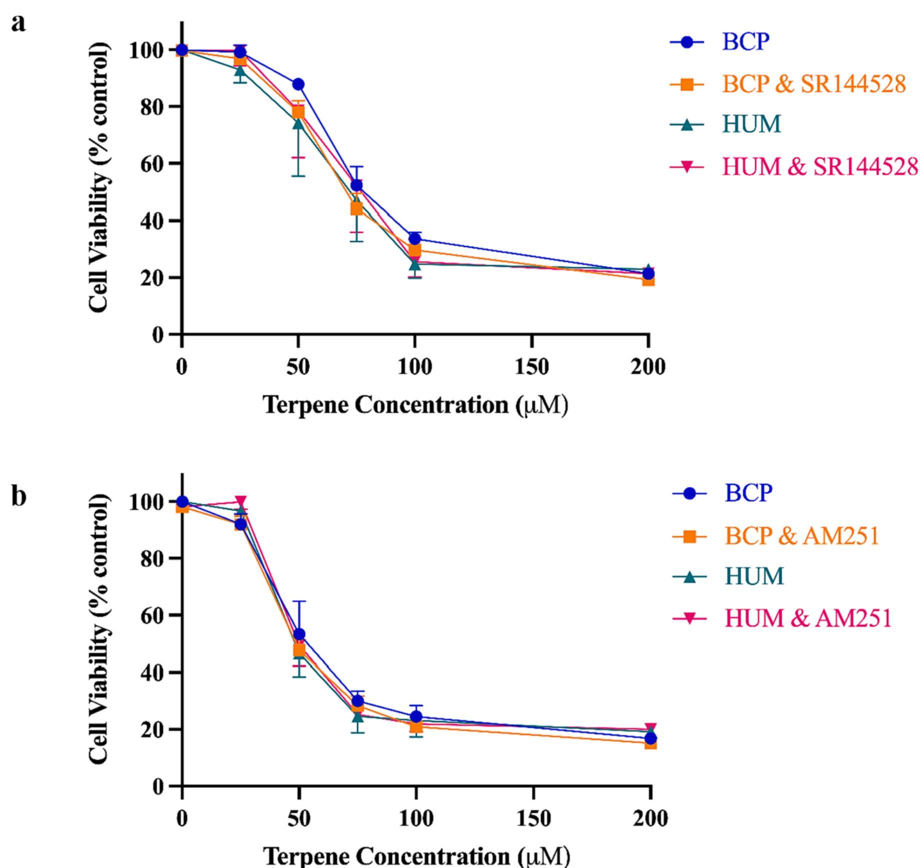

Fig. 4. MTT assay of PC12 neuronal cell viability following 24 hr incubation with BCP and HUM, alone and in the presence of the (**a**) CB2 receptor antagonist SR144528 (100 nM) and (**b**) CB1 receptor inverse agonist AM251 (100 nM). Loss of cell viability to either BCP or HUM was unaffected by CB receptor antagonism (n = 4).

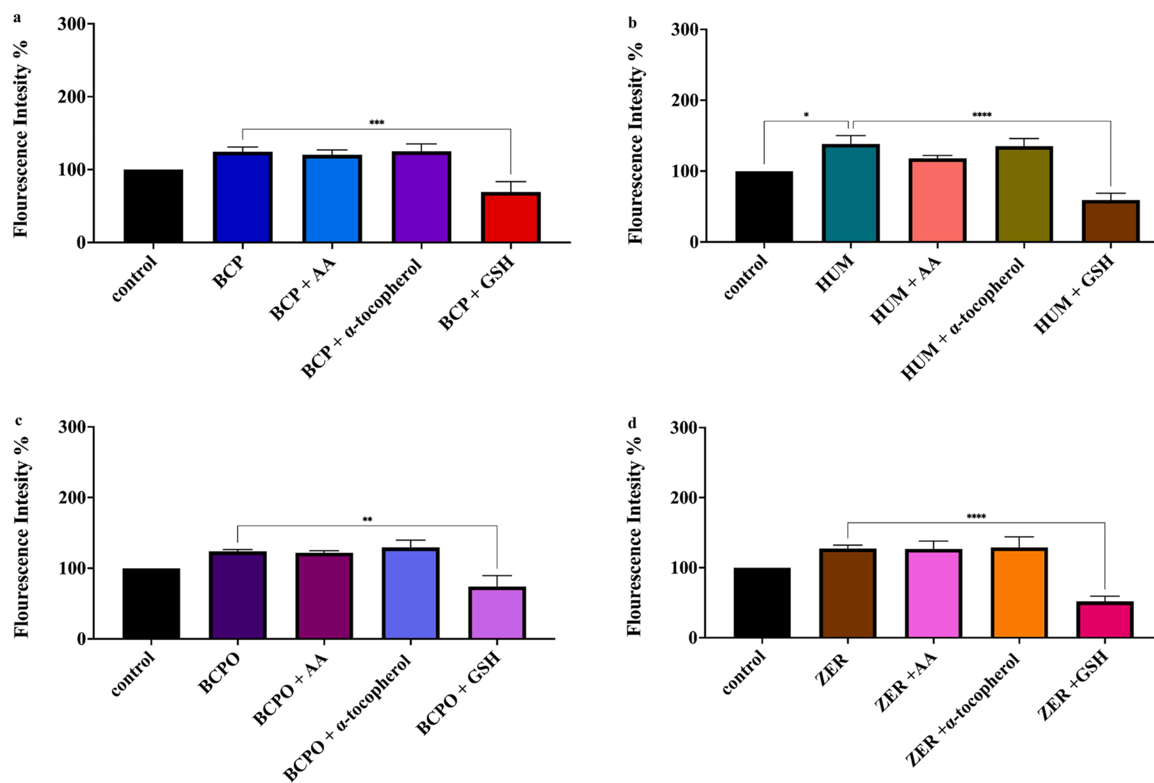

Fig. 5. Effects of sesquiterpenes (**a**) BCP, (**b**) HUM, (**c**) BCPO and (**d**) ZER on intracellular ROS formation in PC12 cells (as % ROS formation) via DCFH-DA assay after 4 hr incubation. PC12 cells were pretreated with ascorbic acid (AA; 50 μM), α-tocopherol (100 μM) or glutathione (GSH; 1 mM) followed by individual sesquiterpene (200 μM) incubation for 4 hrs. Only humulene (HUM) significantly increased baseline ROS after 4 hr incubation (*P $<$ 0.05 vs vehicle). However, GSH significantly reduced ROS formation in the presence of all sesquiterpenes at 4 hrs (**a-d**) (****P $<$ 0.0001, *** P $<$ 0.001, **P $<$ 0.01 vs vehicle).

markers (Menezes et al., 2021). Although limited studies reveal direct antioxidant effects via inhibition of intracellular oxidative stress and indirect antioxidant effects via promotion of antioxidant enzymes for PC12 neuronal cells (Piccialli et al., 2021; Porres-Martinez et al., 2016), the precise neuronal bioactivity profile for these sesquiterpenes is currently lacking.

Overall, in the present study, humulene, zerumbone and β-caryophyllene all demonstrated a concentration-dependent loss of PC12 cell viability after 24-hour incubation. This indicates a consistent neurotoxicity profile for these closely related sesquiterpenes and their oxide forms, with the exception of β-caryophyllene oxide which was relatively benign to PC12 cells overall, causing a comparatively smaller loss of cell viability. The relative lack of loss of PC12 cell viability from β-caryophyllene oxide (BCPO) exposure was unexpected, because it displays stronger proapoptotic activities than BCP from its epoxide exocyclic functional groups (Fidyt et al., 2016). However, BCP may possess more bioactivity then BCPO because BCP is more permeable across cell membranes, thereby potentiating lipid peroxidation and oxidative stress (Sarpietro et al., 2015). Additionally, loss of cell viability from BCPO may be offset by its anti-inflammatory effects, where NLRP3 inflammasome reductions occur from BCPO exposure (Li et al., 2021) and where attenuation of the NLRP3 inflammasome impacts PC12 cell survival (Gong et al., 2018). Based on our findings regarding the differing degree of toxicity exhibited between BCP and BCPO, we would consider it unlikely that oxidation of BCP to BCPO occurs in vitro after 24-hours of incubation, or that any discernible neuronal metabolic biotransformation occurs in this setting.

Terpenes are known to enhance lipid membrane permeability, increase mitochondrial depolarization and elevate reactive oxygen species, leading to increased lipid peroxidation and activation of antioxidant defense systems (Kumari et al., 2019). BCP in particular was previously shown to evoke cell loss via mitochondrial-mediated oxidative stress following 24-hour exposure (Ramachandhiran et al., 2022), however BCP itself was benign up to 25 μM in PC12 cells where it also demonstrated neuroprotective effects from amyloid-evoked neuronal stress (Zhang et al., 2022). Therefore, the potential threshold concentrations for loss of neuronal cell viability from BCP may be less than 25 uM, which was a concentration that was relatively innocuous to PC12 cells in the present study.

Humulene was also found to elicit significant concentration-dependent neurotoxicity and is recognized by its capacity to generate ROS and oxidative stress (Agus, 2021; Richter et al., 2021). It, along with the other major sesquiterpene BCP, has previously shown to be major components of copaiba essential oil responsible for apoptosis in neuronal SH-SY5Y cells (Urasaki et al., 2020) and has notable bioactivity targeting mitochondrial ROS formation and accumulation more generally (Mendes de Lacerda Leite et al., 2021).

In terms of the mitigating presence of cellular antioxidants to cell viability, variable but significant neuroprotection to BCP and HUM-evoked toxicity was evidenced in the presence of α-tocopherol. The neuroprotective activity of α-tocopherol could be from attenuating the reaction of BCP and its open ringed isomer HUM with the superoxide anion, thus preventing lipid peroxidation (Legault et al., 2013). Additionally, HUM can decrease cellular GSH content and increase ROS production to induce cytotoxicity in tumor cell lines (Legault et al., 2003), although no abrogation of toxicity to HUM or BCP was observed with GSH in the present study.

In terms of reactive oxygen species production in the present study, GSH significantly reduced intracellular ROS levels from HUM (138 % to 59 %), ZER (127 % to 52 %), BCP (125 % to 69 %) and BCPO (124 % to 74 %) exposure at 4-hours. Additionally, GSH significantly reduced loss of PC12 cell viability against ZER exposure at 75 μM-200 μM. ZER is a sesquiterpene lactone containing a α,β-unsaturated carbonyl moiety that can provoke accelerated losses of intracellular GSH concentrations by

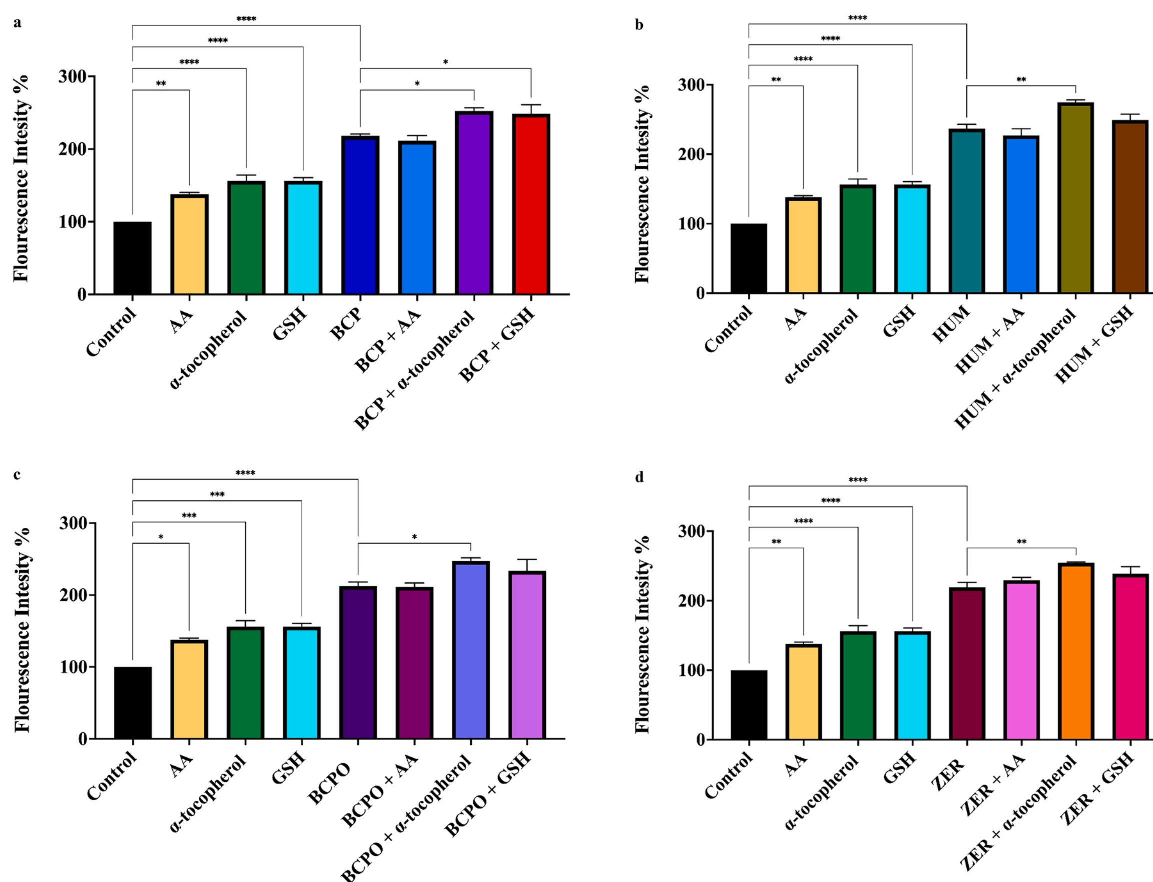

Fig. 6. Effects of sesquiterpenes and antioxidants on intracellular ROS formation in PC12 cells (as % ROS formation) via DCFH-DA assay after longer-term (24 hr) incubation. PC12 cells were pretreated with ascorbic acid (AA; 50 μM), α-tocopherol (100 μM) or glutathione (GSH; 1 mM) followed by (a) BCP, (b) HUM, (c) BCPO or (d) ZER (200 μM) incubation for 24 hrs (a-d). After 24 hr incubation each of the four sesquiterpenes significantly increased ROS formation (Fig. 6a-d; ****$P < 0.0001$ vs Control). Interestingly, after 24 hr exposure, all antioxidants alone significantly increased DCFH-DA fluorescence; in the presence of each sesquiterpene, this was further significantly augmented with α-tocopherol, while GSH significantly increased BCP-evoked ROS formation. ****$P < 0.0001$ ***$P < 0.001$ **$P < 0.01$, *$P < 0.05$ vs vehicle. $n = 4$.

the formation of Michael adducts, triggering elevation of intracellular redox potential (Butturini et al., 2011; Zhang et al., 2005). In our study, the non-thiol-based antioxidants (ascorbic acid and α-tocopherol) had a relative lack of effect on ROS, indirectly supporting the GSH-depleting ability of zerumbone after short-term (4-hour) incubation.

Paradoxically, both antioxidants and terpenes significantly elevated intracellular ROS levels from longer-term (24-hour) exposure as indicated by the DCFDA assay, with none of the antioxidants mitigating ROS generation arising from sesquiterpene exposure. Abnormal intracellular increases in reducing equivalents such as glutathione can cause reductive stress, resulting in increased mitochondrial oxidation and production of ROS (Kornienko et al., 2019). Reductive stress occurs when antioxidant defenses exceed basal or constitutive cellular oxidant production, causing abnormally high levels of reduced species within a biological system (Lloret et al., 2016). Reductive stress can be triggered from an overabundance of antioxidants leading to disturbances in metabolic cues, and overexpression of antioxidant enzymes, thereby inducing metabolic imbalance (Gostner et al., 2015). For example, in HeLa cells, the Vitamin E analog Trolox (2.5 μM – 15 μM) exhibited concentration-dependent antioxidant activity, but at higher concentrations (40 μM – 160 μM) and extended incubation times, Trolox exhibited prooxidant activities as indicated by an increase in ROS levels and reduction in cell viability (Giordano et al., 2020). The authors found that 40 μM is the threshold concentration for Trolox to increase basal ROS due to the accumulation of phenoxy radicals (PhO·).

Analogous to these findings, another study evidenced that at higher concentrations of α-tocopherol, ROS levels increased markedly in lung cancer cells (Lo et al., 2013), collectively suggesting biphasic effects on ROS formation based on exposure time and concentration. Such a dichotomous profile of anti- and pro-oxidative properties may have clinical translation related to toxicity from high-dose vitamin E intake (Pearson et al., 2006). For GSH, a range of pathways may be responsible for its cell toxicity via altered redox status or toxicity arising directly from a range of conjugates (Monks et al., 1990), including glutathione-terpenoid conjugates such as pulegone (Lassila et al., 2016).

Zerumbone also caused concentration-dependent neurotoxicity in PC12 cells which was significantly inhibited in the presence of GSH. HUM, BCP and BCPO lack the α, β-unsaturated carbonyl group found in zerumbone, ascribing it specific bioactivity. For example, zerumbone but not humulene was found to significantly suppress superoxide anions ($O_2^{\cdot -}$) generated from NADPH oxidase in leukocytes, reducing lipopolysaccharide-stimulated expression of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in mouse macrophages, and demonstrated antiproliferative activity in colonic cells (Murakami et al., 2002). In addition, the electrophilic property of zerumbone can significantly induce glutathione S-transferase that conjugate xenobiotics with glutathione, while the non-electrophilic humulene did not show any inducing effects (Nakamura et al., 2004). Furthermore, zerumbone but not humulene can also bind to Kelch-like ECH-associated protein 1 (Keap1) and induce Phase II detoxification enzymes in macrophages, with the α, β-unsaturated carbonyl in zerumbone facilitating formation of thiol ethers (Ohnishi et al., 2009). Keap1 retains 27 cysteine residues

containing a thiol (-SH) group functioning as sensors for oxidative or electrophilic bouts and upon its cysteine alkylation or oxidation, Keap1 unhinges from nuclear factor erythroid 2–related factor 2 (Nrf2) leading to its activation (Matzinger et al., 2018). Various stimuli (exogenous small molecules, ROS, electrophilic or proteotoxic stress) can activate Nrf2, thereby acting as indirect antioxidants by triggering the promotion of antioxidant enzymes and detoxication of xenobiotics (Bellezza et al., 2018; Rahman et al., 2023).

Neuronal cells use GSH to reduce cellular oxidative damage but are depleted in patients with AD (Charisis et al., 2021), while PC12 cells also require GSH for protection against neurotoxicants (Pereira and Oliveira, 1997). GSH is crucial for intracellular redox regulation by maintaining a reduced state of the cysteinyl-thiol groups of proteins and also acts as a coenzyme with glutathione peroxidase (GPx), which utilizes GSH to detoxify H_2O_2, thereby inhibiting necrosis and apoptosis (Guo et al., 2007). This might explain how GSH preserved PC12 cell viability from zerumbone exposure, suggesting that its apoptosis-inducing activity is due to its thiol reactivity and ability to deplete intracellular GSH (Deorukhkar et al., 2015), leading to accumulation of detrimental intracellular ROS levels.

More broadly, because zerumbone has been demonstrated to covalently bind to cellular proteins (Ohnishi et al., 2009), it may cause mild proteo-toxic stress to cells and form GSH adducts, but where the balance sits between such sequalae causing cell protection via hormesis versus apoptosis and autophagy is unclear. Additionally, HUM and ZER can markedly induce heat shock protein 70 (HSP70) expression and prompt the removal of denatured proteins that could potentially aggregate, creating ROS (Ohnishi et al., 2013). However, zerumbone can itself suppress both free radical production and inflammasome induction (Murakami et al., 2002). Adaptive mechanisms to mild biological stressors, such as indirect antioxidants or xenobiotic phytochemicals can promote xenohormesis. However, the "double-edged sword' of redox-active phytochemicals may cause a breakdown of self-defense systems from overexposure. Therefore, future studies are required in pharmacometrics and therapeutic dosages of phytochemicals targeting neurogenerative diseases to extend these preclinical studies into clinically-relevant settings where terpenoids may have bioactivity, particularly in settings where botanical extracts or other complex natural product extracts may be prone to oxidation of their principal bioactives including predominant terpenes such as BCP (Sköld et al., 2006), and such as occurs in medicinal cannabis preparation and storage (Al Ubeed et al., 2022).

Conclusion

Specific terpenes, the sesquiterpenes β-caryophyllene, humulene, and zerumbone demonstrated neurotoxic effects in PC12 neuronal cells, while β-caryophyllene oxide was less toxic. The antioxidant α-tocopherol was variably able to alleviate the concentration-dependent loss of PC12 cell viability from exposure to β-caryophyllene and humulene and to a lesser extent, zerumbone, while glutathione inhibited the loss of PC12 cell viability from exposure to zerumbone. Glutathione significantly lessened intracellular neuronal ROS production in combination with each of sesquiterpenes from short-term (4-hour) exposure, but not 24-hour exposure. Over 24-hour exposure, sesquiterpenes and antioxidants markedly increased ROS levels, both individually and in combination, suggesting a type of reductive stress in the presence of antioxidants further augmented with sesquiterpene-based oxidative stress. Future studies of sesquiterpene properties and activities are imperative for evaluating their safety as therapeutic agents targeting neurogenerative diseases.

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CRediT authorship contribution statement

John Staton Laws III: Methodology, Validation, Formal analysis, Data curation, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Scott D. Smid:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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